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E1A Gene Expression Induces Susceptibility to Killing by NK Cells Following Immortalization but Not Adenovirus Infection of Human Cells[1]

JOHN M. ROUTES*,†,‡,[2] and JAMES L. COOK*,†,‡,¶,‖

*Department of Medicine and ¶Robert W. Lisle Laboratory in Immunology and Tumor Cell Biology, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206; and Departments of †Medicine, ‖Microbiology, and ‡Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Adenovirus (Ad) infection and E1A transfection were used to model changes in susceptibility to NK cell killing caused by transient vs stable E1A expression in human cells. Only stably transfected target cells exhibited cytolytic susceptibility, despite expression of equivalent levels of E1A proteins in Ad-infected targets. The inability of E1A gene products to induce cytolytic susceptibility during infection was not explained by an inhibitory effect of viral infection on otherwise susceptible target cells or by viral gene effects on class I MHC antigen expression on target cells. This differential effect of E1A expression on the cytolytic phenotypes of infected and stably transfected human cells suggests that human NK cells provide an effective immunologic barrier against the *in vivo* survival and neoplastic progression of E1A-immortalized cells that may emerge from the reservoir of persistently infected cells in the human host.

INTRODUCTION

Group C adenoviruses (Ad)[3] are common human pathogens with a propensity to cause persistent infections. These viruses can also immortalize mammalian cells from several species including man. Despite their ubiquity, persistence, and competence for cellular immortalization, existing data indicate that human Ad are not oncogenic in their natural host (Green *et al.*, 1979).

One factor that could determine the outcome of an incipient tumor following a transforming viral infection is the competence of the host cellular immune response to eliminate immortalized cells. Previous studies using hamster and rat cells showed that the expression of only the E1A gene following either viral infection or stable transfection is sufficient to induce susceptibility to lysis by host natural killer cells (Cook *et al.*, 1986, 1987, 1989). Furthermore, in studies using E1A-transfected BHK-21 sarcoma cells, there was a strong correlation between E1A induction of the cytolytic susceptible phenotype *in vitro* and NK-cell-dependent tumor rejection *in vivo* (Cook *et al.*, 1993; Walker *et al.*, 1991). In these *in vivo* studies, the host NK cell response could eliminate large numbers (at least 10^7 cells) of cytolytic susceptible, E1A-expressing tumor cells in the absence of T-cell-dependent immune responses.

In contrast to the observations with E1A-expressing, Ad2/5-infected rodent cells which are highly susceptible to lysis by both rodent and human NK cells, Ad2/5 infection with subsequent high-level E1A expression in human cells does not induce increased susceptibility to NK cell killing, irrespective of the donor source of the killer cell population (Routes and Cook, 1989). These data suggest that E1A induction of cytolytic susceptibility seen in rodent cells may be lacking in human cells. Because NK cells appear to be an important component of the antiviral cellular immune response (Welsh, 1986), the failure of E1A to target Ad-infected human cells for elimination by NK cells may contribute to the predilection of Ad to cause persistent infections in man (Routes and Cook, 1989). However, if E1A gene products are incapable of rendering human cells susceptible to NK-cell-mediated killing, E1A-immortalized cells should also escape destruction by the NK cell response. Therefore, if findings from rodent models are applicable to human disease, it would be predicted that Ad would not only cause persistent infections, but also should be oncogenic in man, which is contrary to Ad's known biological behavior.

During studies of induction of cytolytic susceptibility by Ad2/5 E1A gene products in Ad-transformed rodent cells, we observed that the E1A-expressing human embryonic kidney (HEK) cell line 293 is also highly susceptible to lysis by NK cells (Cook *et al.*, 1986). However, this observation was limited to a single cell line, and the hypothesis it suggested regarding E1A function during immortalization of human cells could not be tested using

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[2] To whom correspondence and reprint requests should be addressed at National Jewish Center for Immunology and Respiratory Medicine, Dept. Medicine, 1400 Jackson St., Denver, CO 80206.

[3] Abbreviations used: Ad, adenovirus(es); Ad2/5, adenovirus serotype 2 or 5; HEK, human embryonic kidney.

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a matched, E1A-negative target cell. Based upon this preliminary result and our subsequent report of NK resistance of Ad-infected human cells (Routes and Cook, 1989), we postulated that E1A expression in human cells results in different NK cytolytic phenotypes depending upon whether E1A is expressed during viral infection (in which case the cells are NK resistant) or during stable E1A expression as would occur following immortalization (in which case the cells would be NK susceptible). The present studies were done to determine whether the cytolytic susceptible phenotype of 293 cells is cell-line-specific and to directly test the hypothesis that there is a difference in the ability of E1A to induce cytolytic susceptibility in infected vs stably transfected human cells. The data show that E1A-expressing, immortalized human cells, other than 293, also exhibit high-level cytolytic susceptibility. Furthermore, clonal target cell populations stably expressing E1A following transfection also exhibit cytolytic susceptibility, whereas the same cells remain cytolytic resistant during viral infection despite high-level E1A protein expression. Other data indicate that this lack of induction of cytolytic susceptibility during viral infection of human cells is not caused by either inhibitory effects of viral infection or viral alteration of class I MHC antigen expression on target cells.

MATERIALS AND METHODS

Cell lines

HE1-E1 (Chang *et al.,* 1990) and 293 (Graham *et al.,* 1977) are Ad5-transformed HEK cell lines, derived in different laboratories, that express E1A and E1B genes. A549 is a lung carcinoma cell line. H4 is a subclone of the HT1080 fibrosarcoma cell line (Frisch, 1991). P2AHT2A is an Ad5 E1A-transfected H4 cell line (Frisch, 1991). A2058 is a melanoma cell line (Frisch, 1991). 1A58C8-1 is an Ad5 E1A-transfected A2058 cell line (Frisch, 1991). Jurkat is an Ad-permissive, T cell lymphoma cell line (Lavery *et al.,* 1987). NK3.3 is a cloned NK cell line (Kornbluth *et al.,* 1982). W162 is a Vero cell line which expresses adenovirus type 2 E4 proteins (Weinberg and Ketner, 1983). All cell lines, with the exception of W162, are of human origin. NK3.3 was maintained in RPMI 1640 supplemented with 15% fetal calf serum, 15% interleukin-2 medium (Lymphocult-T, Biotest Diagnostics Corp., Fairfield, NJ), antibiotics (penicillin, 100 u/ml; streptomycin, 100 mg/ml), and glutamine (2 m*M*). Primary HEK cells, A549, 293, HE1-E1, H4, P2AHT2A, A2058, 1A58C18, W162, and Jurkat were maintained in DMEM supplemented with 5 to 10% fetal calf serum, glucose (15 m*M*), antibiotics, and glutamine. All cell lines were tested for contamination by Mycoplasma by the Mycotect assay (Bethesda Research Laboratories, Gaithersburg, MD) and were negative. NK3.3 was obtained from Jacki Kornbluth (University of Arkansas, Little Rock). A549 and 293 were obtained from the American Type Culture Collection (Rockville, MD). HE1-E1 was obtained from L. S. Chang (Ohio State University, Columbus). Jurkat was obtained from Dan Lavery (Mt. Sinai School of Medicine, New York). Primary HEK cells were obtained from Whitaker Biosciences (Walkersville, MD). H4, P2AHT2A, A2058, and 1A58C18 were provided by Steve Frisch (La Jolla Cancer Research Center, La Jolla, CA).

Viruses

H5dl339 (Logan *et al.,* 1984) is an E1B deletion mutant that does not express either the 19- or 55-kDa proteins. H5dl802 (Rice and Klessig, 1985) is an E2 deletion mutant that does not express the 72-kDa DNA-binding protein. H5dl1011 (Bridge *et al.,* 1993) is an E4 deletion mutant that does not express any of the six proteins encoded within E4. H2dl801 (Challberg and Ketner, 1981) is an Ad2 E3 deletion mutant that does not express any E3 proteins, whereas H5dl309 (Jones and Shenk, 1979) is an Ad5, E3 deletion mutant that does not express the E3 10.4-, 14.5-, or 14.7-kDa proteins (Gooding *et al.,* 1991). Wild-type Ad5, H2dl801, and H5dl309 were grown in A549 cells. H5dl339 was grown in 293 cells. H5dl1011 was grown W162 cells. All viruses were titered by plaquing in the cell line in which they were grown.

Cytolysis assays

Prior to the cytolysis assays, control experiments were performed using all the viral mutants to ensure that E1A expression in A549 cells was comparable to that obtained following wild-type Ad5 infection. NK cytolysis assays were performed as previously described (Cook *et al.,* 1987). Briefly, Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ)-separated peripheral blood mononuclear cells or NK3.3 cells served as the sources of NK cells as indicated. Target cells were infected with Ad5, H5dl309, H5dl339, H2dl801 (100 PFU/cell), H5dl802, or H5dl011 as described (Cook *et al.,* 1987) for 16 hr (A549, 293, HE1-E1, H4, P2AHT2A, A2058, 1A58C18) or 40 hr (Jurkat) and labeled with [^{51}Cr] (100 mCi/ml for 1 hr; 1 Ci = 37 gBq). NK cells and target cells (1×10^4 cells) were cosedimented at different effector to target cell (E:T) ratios in flat-bottom microtiter plates by centrifugation at 500 *g* for 3 min, and after a 6- or 18-hr incubation at 37° in 5% CO_2, half of the supernatant from each well was harvested and counted in a gamma counter. NK cell killing was determined by calculating the percentage of NK cell-induced release of radiolabel from target cells as described (Cook *et al.,* 1987). The results shown in Figs. 1 through 4 represent the mean ± SEM of at least four separate experiments. The mean percentage spontaneous release from all target cells, including those infected with Ad, was less than 20%. The significance of the differences in NK cell killing of matched sets of control, Ad5-infected, and E1A-transfected targets was estimated using the Student *t* test.

Western analysis of E1A proteins

Plates (60 mm) of cells were lysed in RIPA buffer (1% nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 m*M* Tris, pH 7.4, 150 m*M* NaCl), and protein concentrations of RIPA supernatants were determined by the BCA protein assay (Pierce, Rockford, IL). An equal amount of protein from each cell lysate was separated on 10% SDS polyacrylamide gels and electrophoretically transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 5% nonfat milk solution and incubated with the anti-E1A monoclonal antibody M73 (Harlow *et al.*, 1985) supplied by E. Harlow (Massachusetts General Hospital, Charleston, MA) for 1 hr. Following several washes with PBST (PBS with 0.05% Triton X-100), the membranes were incubated for 1 hr with rabbit anti-mouse antibody (Cappel, Durham, NC) and washed extensively with PBST, followed by an additional incubation with ^{125}I-labeled protein A (ICN, Costa Mesa, CA). Following several washes with PBST, the bands were visualized by fluorography.

RESULTS

Susceptibility of Ad-transformed, E1A-expressing HEK cells to lysis by NK cells

Most of the NK-susceptible target cells that have been reported in the literature, whether of human or other species origin, are derived from spontaneous malignancies and do not express any known viral antigens. Viral gene expression is clearly not *necessary* for expression of the cytolytic susceptible phenotype. Therefore, it was possible that the cytolytic susceptibility that we observed previously using 293 as target cells in NK cell cytolysis assays (Cook *et al.*, 1986) was caused by a cell-line-specific peculiarity of 293 cells that is not characteristic of other E1A-expressing, Ad5-transformed human cells. To test this hypothesis, an independently derived line of Ad5-transformed HEK cells, HE1-E1, established after transfection with the Ad5 E1 gene region (containing the E1A and E1B genes), was compared to 293 cells for susceptibility to NK killing. Ad5-infected A549 cells were used as E1A-expressing, cytolytic resistant controls (Routes and Cook, 1989).

Like 293, HE1-E1 target cells were highly susceptible to lysis by human NK cells (Fig. 1). These data indicated that the association between E1A expression and cytolytic susceptibility in human cells is not unique to the 293 cell line and suggested the hypothesis that constitutive E1A expression following immortalization of other inherently cytolytic resistant, human cells would convey the cytolytic susceptible phenotype.

The use of HEK cells as target cells to compare to 293 and HE1-E1 to test this hypothesis was problematic for two reasons. First, 293 and HE1-E1 represent the results of high-level cell selection during transformation from heterogeneous, uncloned populations of transfected HEK cells. Therefore, comparisons of 293 and HE1-E1 with different lots of HEK cells might not be representative of the effects of E1A expression but might be an artifact of target cell selection. Second, it was not possible to control or adequately test different lots of HEK cells for contamination with adventitious agents that might confound the interpretation of NK cell cytolysis assays by making otherwise cytolytic resistant cells appear susceptible to lysis (Brooks *et al.*, 1979). These problems, coupled with our observations that different HEK cell lots obtained commercially exhibited variable resistance to killing by human NK cells (data not shown), indicated that another approach was needed to model comparisons of the effects of E1A expression on virally infected and E1A-immortalized target cells.

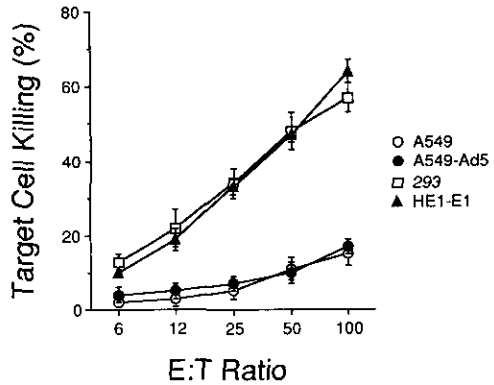

FIG. 1. Human NK cell killing of Ad5-transformed (293, HE1-E1) or Ad5-infected human cells. NK cell killing (mean ± SEM of four experiments) of lung carcinoma cells (A549), Ad5-infected A549 (100 PFU/cell for 16 hr), 293, and HE1-E1 cells in 6-hr cytolysis assays. Peripheral blood mononuclear cells served as the source of NK cells for these assays.

Comparison of the NK cytolytic phenotypes of Ad5 E1A-transfected and Ad5-infected human target cells from the same clonal populations

To examine the cytolytic susceptibilities of the same human target cells expressing E1A proteins either during Ad infection or after E1A transfection, two human tumor cell lines (a fibrosarcoma and a melanoma) were compared, before and after Ad infection, with their E1A-transfected derivative cell lines. These two parental cell lines were chosen for their inherent resistance to lysis by human NK cells, their permissiveness for Ad5 infection (and E1A expression), and their susceptibility to E1A gene transfection. When these sets of target cells were tested for susceptibility to NK killing, they exhibited patterns of cytolytic phenotypes identical to those observed with the Ad5-transformed HEK cell lines and Ad5-infected A549 cells (Fig. 2). Specifically, E1A-expressing, Ad5-infected fibrosarcoma cells (H4-Ad5) and melanoma cells (A2058-Ad5) remained highly resistant to NK killing,

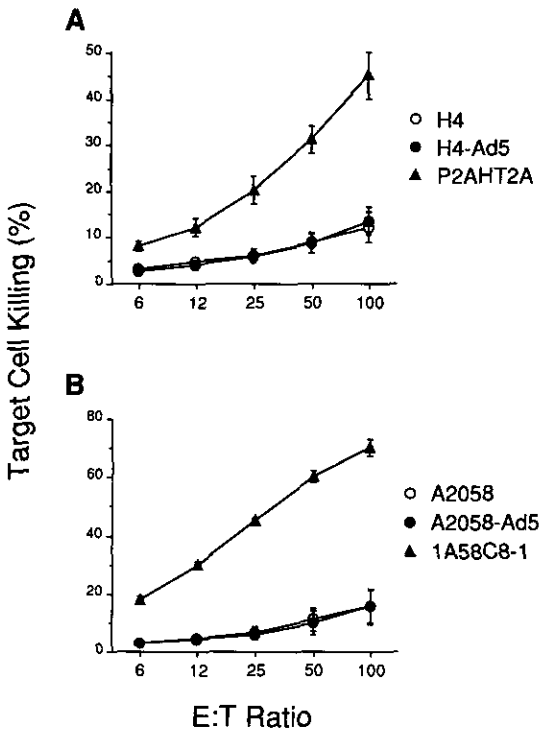

FIG. 2. Human NK cell killing of Ad5 E1A-transfected versus Ad5-infected human cells in 6-hr cytolysis assays. (A) NK cell killing of E1A-transfected H4 fibrosarcoma cells (P2AHT2A) and mock (H4)- or Ad5-infected H4 cells (H4-Ad5). (B) NK cell killing of E1A-transfected A2058 cells (1A58C8-1) and uninfected (A2058) or Ad5-infected A2058 cells. Peripheral blood mononuclear cells served as the source of NK cells for these assays.

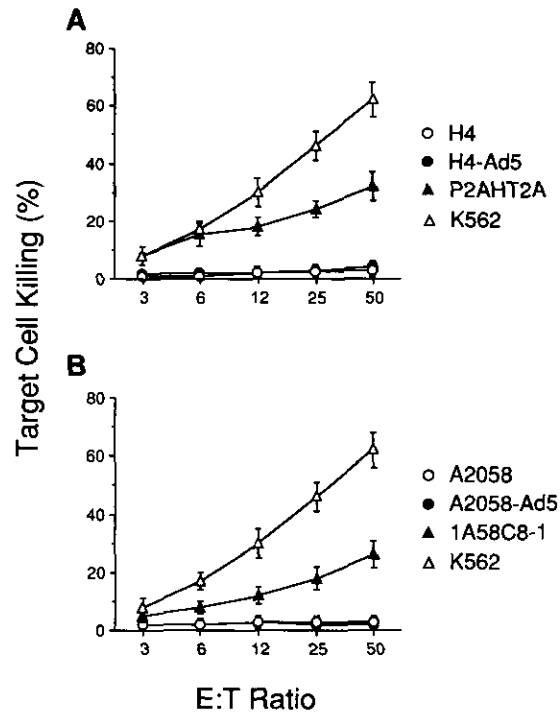

FIG. 3. Killing of Ad5 E1A-transfected versus Ad5-infected fibrosarcoma or melanoma cells by the cloned human NK cell line NK3.3. (A) NK cell killing of H4, Ad5-infected H4 cells, or P2AHT2A. (B) NK cell killing of A2058, Ad5-infected A2058 cells, or 1A58C8-1. K562 cells were used as the cytolytic susceptible control in these 6-hr cytolysis assays.

whereas the E1A-transfected clones derived from each cell line (P2AHT2A and 1A58C18-1, respectively) had become highly susceptible to NK cell-induced lysis. These results support the hypothesis that stable expression of E1A proteins in human cells, such as is observed after cellular immortalization, induces high-level cytolytic susceptibility to NK cells, whereas E1A protein expression in the context of viral infection of human cells does not.

In the cytolysis assays presented to this point, freshly isolated blood mononuclear cells were used as the source of NK cells. To confirm that human NK cells can discriminate between Ad-infected and E1A-immortalized human target cells in the absence of any other mononuclear cell or blood components in these preparations, cytolysis assays were repeated with the same matched sets of control and E1A-expressing target cells represented in Fig. 2 using the cloned, human NK cell line NK3.3 (Kornbluth *et al.*, 1982) as the killer cell population (Figs. 3 and 4). The prototypic, NK-susceptible target cell line K562 was used as a highly susceptible control for NK3.3 cytolytic activity. These NK3.3 cytolysis assays produced exactly the same patterns of target cell killing observed using fresh, blood NK cells. In 6-hr cytolysis assays (Fig. 3), Ad5-infected fibrosarcoma (H4) and melanoma (A2058) cells remained resistant to lysis by NK3.3

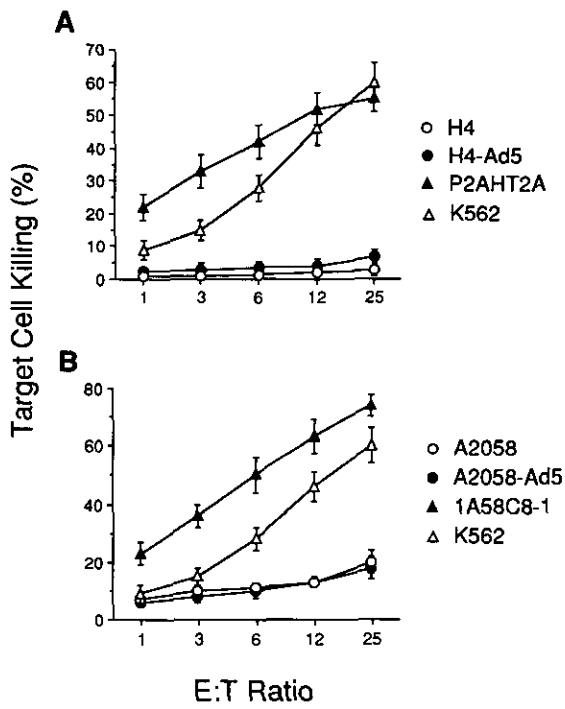

FIG. 4. Differential NK3.3 cell killing of E1A-transfected human cells compared to Ad5-infected cells in 18-hr cytolysis assays. (A) NK cell killing of H4, Ad5-infected H4 cells, or P2AHT2A. (B) NK cell killing of A2058, Ad5-infected A2058 cells, or 1A58C8-1.

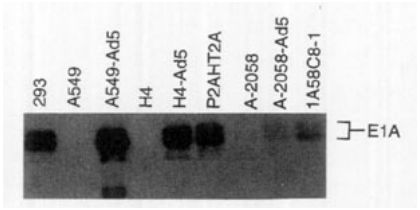

FIG. 5. E1A protein levels in Ad5-infected, Ad5-transformed, or E1A-transfected human cells. Steady-state levels of E1A proteins as measured by Western analysis in P2AHT2A, 1A58C8-1, or Ad5-infected H4 or A2058 cells (100 PFU/cell, 16 hr). No reproducible differences were seen in E1A protein expression in matched, Ad5-infected vs E1A-transfected cell lines (Ad5-infected A2058 vs 1A58C8-1; Ad5-infected H4 vs P2AHT2A).

killer cells, whereas the E1A-transfected derivative cell lines (P2AHT2A and 1A58C18-1, respectively) exhibited increased cytolytic susceptibility. The amplitude of killing of the E1A transfectants was lower than that detected against K562 controls and was also lower than had been observed using blood NK cells in 6-hr cytolysis assays (compare Figs. 2 and 3). When the length of the NK3.3 cytolysis assays was prolonged from 6 to 18 hr, these differences in amplitude of killing between E1A-expressing, transfected targets and K562 disappeared (Fig. 4). However, despite the prolongation of the cocultivation period of NK3.3 cells and target cells, there was no increase in cytolytic susceptibility observed with virally infected, E1A-expressing fibrosarcoma cells or melanoma cells. These data support the hypothesis that within the same human cell population, human NK cells can discriminate between target cells stably expressing E1A proteins and targets expressing E1A in the context of viral infection.

Comparative expression of E1A gene products in Ad-infected and E1A-transfected target cells

Previous studies using rodent cells showed that E1A induction of NK cell cytolytic susceptibility in Ad2/5-infected and transformed targets is dependent on the level of expression of E1A proteins (Cook *et al.*, 1987, 1989, 1993). Cells expressing E1A at levels below those usually seen during viral infection remain resistant to NK killing, whereas cells expressing E1A at levels comparable to those seen in virally infected or transformed rodent cells become susceptible to lysis by NK cells. E1A expression level was also the determining factor in the ability of the host NK cell response to eliminate E1A-transfected hamster sarcoma cells *in vivo* (Cook *et al.*, 1993). Based upon these observations, we compared E1A protein expression in virally infected versus stably transfected H4 and A2058 cells to determine whether the cytolytic resistance of infected cells was caused by insufficient E1A protein expression. Using quantitative Western analysis (Fig. 5), E1A protein expression level in the transfected cell lines (P2AHT2A, 1A58C18-1) was approximately equivalent to the levels of E1A expression detected in the respective Ad5-infected target cells (H4 or A2058). In fact, the highest level of E1A protein expression observed in these studies was that seen with Ad5-infected A549 cells (Fig. 5), which are consistently and highly resistant to NK killing (Fig. 1). These results indicate that E1A induction of cytolytic susceptibility in human target cells is correlated more closely with the conditions under which E1A is expressed (i.e., infection or immortalization) than with the absolute levels of E1A protein expression. These results do not exclude the possibility suggested by the rodent cell studies that relatively low levels of E1A expression during stable transfection of human cells (Cook *et al.*, 1993) would also fail to induce the cytolytic susceptible phenotype in these target cells.

Role of viral infection in inducing cytolytic resistance

An obvious difference in the conditions of the two sets of target cells contrasted in these studies was that one set was undergoing active viral infection. Therefore, it is possible that adenoviral gene products other than E1A actively blocked the susceptibility of target cells to NK cell killing. The two most likely candidates, the E1B and E3 gene products, which reduce susceptibility mediated by TNF-α (E1B: 19-kDa protein; E3: 10.4-, 14.5-, and 14.7-kDa proteins) (Gooding *et al.*, 1988, 1991) or diminish apoptotic cell death caused by E1A protein expression (E1B: 19-kDa protein) (Rao *et al.*, 1992; White and Stillman, 1987), were not found to inhibit the cytolytic susceptible phenotype. Both 293 and HE1-E1 express large amounts of the E1B 55- and 19-kDa proteins (data not shown) and are highly susceptible to NK cell killing (Fig. 1), observations consistent with our previous studies showing the inability of E1B expression to block NK killing of Ad-transformed, E1A-expressing rodent target cells (Cook *et al.*, 1993). Furthermore, infection of A549 cells with the E1B deletion mutant H5dl339, which does not express either the 19- or 55-kDa proteins, did not result in increased killing of A549 cells in comparison to uninfected control cells (Table 1).

To address the role of E3 gene products in blocking the cytolytic susceptible phenotype, A549 cells were infected with either H2dl801, which contains a deletion of the entire E3 coding region (Challberg and Ketner, 1981), or H5dl309 (Jones and Shenk, 1979), which does not express the E3 10.4-, 14.5-, or 14.7-kDa proteins (Gooding *et al.*, 1988). There was no increase in NK cell killing of A549 cells infected with either H5dl309 or H2dl801 compared to uninfected cells (Table 1). Similar patterns of killing were also obtained when A549 cells were infected with H5dl802 or H5dl1011, which contain deletions in the E2 (Rice and Klessig, 1985) or E4 (Bridge *et al.*, 1993) genes, respectively (Table 1). The failure of the Ad E1B, E2, E3, or E4 mutants to induce cytolytic susceptibility in A549 cells was not secondary to inade-

TABLE 1

NK Cell Killing of A549 Cells Infected with Mutant Adenoviruses[a]

Virus infection	Mutated gene	Target cell killing (%)
None	NA	15 ± 1.7
H5dl339	E1B	16 ± 2
H5dl802	E2	17 ± 2
H2dl1011	E4	17 ± 2
None	NA	7.6 ± 1
H5dl309	E3	6.0 ± 1
H2dl801	E3	6.7 ± 1

[a] The results (mean ± SEM percentage target cell killing) of three cytolysis assays at the optimal 100:1 blood mononuclear to target cell ratio. Experiments performed together are grouped above.

quate E1A expression. E1A protein expression in A549 infected with these viral mutants was equivalent to that measured using wild-type Ad5 (data not shown). There are several other viral genes and cellular consequences of viral infection (e.g., endogenous effects on cellular function and exogenous effects such as cytokine production) which could conceivably block the susceptibility of target cells to NK cell killing. A simple and direct method to test whether Ad5 infection inhibits target cell killing is to infect target cells that are both inherently cytolytic susceptible and permissive for Ad5 infection. The E1A-expressing cells 293, HE1-E1, 1A58C8-1, and P2AHT2A and the E1A-negative, lymphoma cell line Jurkat (Lavery *et al.*, 1987) satisfy these criteria and were used for these experiments. There were no significant differences in the percentage target cell killing (i.e., neither decreased or increased killing) of these two types of target cells after infection with Ad5 (Table 2).

These data disprove the hypothesis that viral infection or the E1B, E2, E3, or E4 gene products are responsible for blocking E1A induction of cytolytic susceptibility in virally infected human cells.

DISCUSSION

Adenoviruses are common human pathogens with a propensity to cause persistent infections. For example, over 95% of adults possess antibodies directed against one or more of group C Ad serotypes alone (Straus, 1984), and continued excretion of virus has been documented for as long as 24 months after acquisition of the original viral infection (Fox *et al.*, 1969). For reasons that are poorly understood, Ad appear to be nononcogenic in man, even though they are competent to transform human cells and some serotypes are oncogenic in other species.

Clinical studies demonstrate that cellular immunity is of central importance in preventing disseminated or fatal Ad infections (Koneru *et al.*, 1987; Lederman and Winkelstein, 1985; Wasserman *et al.*, 1988; Zahradnik *et al.*, 1980). Because NK cells are an important component of cellular antiviral immunity in general and provide a critical immunologic defense against Ad2/5 E1A-expressing tumor cells in rodents (Cook *et al.*, 1993; Walker *et al.*, 1991), we postulated that NK cells would be important in the clearance of both Ad-infected and Ad-transformed cells in man. However, our initial studies showed that in human cells, E1A gene products produced in the context of Ad infection are unable to induce cytolytic susceptibility to NK cells (Routes and Cook, 1989), suggesting that NK cells may be impaired in their ability to mediate anti-adenoviral immunity. Experiments reported here clarify the possible role of NK cells in eliminating Ad-immortalized contrasted with Ad-infected cells. Clear differences in the capacities of NK cells to selectively kill either Ad-infected or constitutively E1A-expressing human cells were shown. Cells expressing high levels of E1A proteins during viral infection remained resistant to NK killing, whereas the same cells became highly susceptible to NK killing when stably expressing E1A proteins after stable transfection (P2AHT2A, 1A58C18-1) or transformation (293, HE1-E1).

The molecular basis for the difference in NK cell cytolytic susceptibility of Ad-infected compared to Ad-immortalized human cells is unknown. In other experimental models, reduction in the expression of class I MHC antigen on the target cell surface correlates with increased susceptibility to NK cell lysis (Kärre *et al.*, 1986). This correlation between cytolytic susceptibility and MHC expression has not been found in our studies of Ad2/5-infected and Ad-immortalized human cells. We previously reported that the E3 19-kDa gene product decreases surface class I MHC antigen expression following 16 hr Ad5-infection of 293, A549 (Routes and Cook, 1990), and H4 (Routes *et al.*, 1993) cells by approximately 70, 25, and 15%, respectively, with a 50% reduction on Jurkat

TABLE 2

NK Cell Lysis of Uninfected and Ad5-Infected, Cytolytic Susceptible Cell Lines[a]

Cell line	Target cell killing (%)
293	40 ± 5
293-Ad5	41 ± 5
Hel-El	77 ± 5
Hel-El-Ad5	73 ± 3
1A58C8-1	68 ± 4
1A58C8-1-Ad5	71 ± 5
Jurkat	72 ± 6
Jurkat-Ad5	76 ± 8
P2AHT2A	50 ± 5
P2AHT2A-Ad5	49 ± 7

[a] The results (mean ± SEM percentage target cell killing) of three cytolysis assays at 100:1 blood mononuclear to target cell ratio.

cells following 40 hr of infection. However, these Ad-induced reductions in class I MHC expression do not positively or negatively affect the cytolytic susceptibilities of these different target cells (Figs. 1–4 and Table 2). P2AHT2A, the E1A-transfected cell line derived from H4 fibrosarcoma cells, exhibited markedly *increased* cytolytic susceptibility compared to H4 (Figs. 2, 3, 4), despite the fact that P2AHT2A expresses 50% *more* class I MHC Ag than H4 (Routes *et al.,* 1993). Furthermore, Ad5 infection of P2AHT2A decreases class I MHC Ag expression by approximately 70% (Routes *et al.,* 1993) without altering its cytolytic susceptibility to NK cell lysis (Table 2).

Previous reports document that in stably transfected rodent cells, Ad2/5 E1A gene expression is both necessary and sufficient to induce cytolytic susceptibility (Cook *et al.,* 1986, 1987). Data presented here showed that Ad infection of human cells did not alter the baseline level of cytolytic susceptibility, regardless of whether the cell was cytolytic resistant (A549, H4, A2058) or susceptible (Jurkat, 293) prior to infection. These data suggest that the increased cytolytic susceptibility of E1A-immortalized and transfected human target cells contrasted with Ad-infected human cells is a result of qualitative or quantitative changes in the effects of E1A proteins in human cells that occur during the immortalization process. Reported functional differences of E1A proteins in infected compared to transformed cells include differences in half lives (Spindler and Berk, 1984), transcriptional activation of E1A-inducible genes (Alwine, 1985; Leff and Chambon, 1986; Martin *et al.,* 1990; Svensson and Akusjarvi, 1984), colocalization with cellular heat shock protein (White *et al.,* 1988), and effects on host cell cytoskeletal structure (White *et al.,* 1988). Whether any of these differences in expression or E1A protein effects on human cells explain the enhanced cytolytic susceptibility of E1A-immortalized cells remains to be determined. Any hypothesis to explain increased cytolytic susceptibility of immortalized cells and the lack of cytolytic susceptibility of infected human cells must also account for the observation that infected hamster and rat cells expressing E1A proteins are, in contrast to infected human cells, NK susceptible (Cook *et al.,* 1987).

We hypothesize that these results help explain the seemingly paradoxical biological behavior of Ad infection in man. Ad infection and persistence may have evolved partly due to the inability of E1A expression to render infected cells susceptible to NK cell attack. This adaptive mechanism may complement other strategies attributed to Ad that undermine man's antiviral immune response (Anderson and Fennie, 1987; Burgert *et al.,* 1987; Gooding *et al.,* 1988, 1991; Kitajewski *et al.,* 1986). However, despite the relative incompetence of NK cells to mediate anti-adenoviral immunity, the results reported here indicate that human NK cells provide an effective immunologic defense against the *in vivo* survival of E1A-immortalized cells. This E1A-induced susceptibility of immortalized cells to the antineoplastic effects of NK cells could complement other antioncogenic effects attributed to E1A (Frisch, 1991) to prevent the emergence of neoplastic cells from populations of human cells chronically infected with Ad.

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REFERENCES

Alwine, J. (1985). Transient gene expression control: Effects of transfected DNA stability and *trans*-activation by viral early proteins. *Mol. Cell. Biol.* **5,** 1034–1042.

Anderson, K. P., and Fennie, E. H. (1987). Adenovirus early region 1A modulation of interferon antiviral activity. *J. Virol.* **61,** 787–795.

Bridge, E., Medghalchi, S., Ubol, S., Leesong, M., and Ketner, G. (1993). Adenovirus early region 4 and viral DNA synthesis. *Virology* **193,** 794–801.

Brooks, C. G., Rees, R. C., and Leach, R. H. (1979). High nonspecific reactivity of normal lymphocytes against mycoplasma-infected target cells in cytotoxicity assays. *Eur. J. Immunol.* **9,** 159–165.

Burgert, H. G., Maryanski, J. L., and Kvist, S. (1987). "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigen. *Proc. Natl. Acad. Sci. USA* **84,** 1356–1360.

Challberg, S., and Ketner, G. (1981). Deletion mutants of Ad2: Isolation and initial characterization of viruses carrying mutations near the right end of the viral genome. *Virology* **114,** 196–209.

Chang, L.-S., Martina, J. D., Levine, A. J., and Shenk, T. E. (1990). "Suppression of transformation of SV40 T- and adenovirus E1-transformed human cells by the wild-type human RB and p53 genes." SV40, Polyoma, and Adenoviruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, August 15–19, 1990.

Cook, J. L., May, D. L., Lewis, A., Jr., and Walker, T. A. (1987). Adenovirus E1A gene induction of susceptibility to lysis by natural killer cells and activated macrophages in infected rodent cells. *J. Virol.* **61,** 3510–3520.

Cook, J. L., May, D. L., Wilson, B. A., Holskin, B., Chen, M.-J., Shalloway, D., and Walker, T. A. (1989). Role of tumor necrosis factor-alpha in E1A oncogene-induced susceptibility of neoplastic cells to lysis by natural killer cells and activated macrophages. *J. Immunol.* **142,** 4527–4534.

Cook, J. L., Walker, T. A., Lewis, A. M., Jr., Ruley, H. E., Graham, F. L., and Pilder, S. H. (1986). Expression of the adenovirus E1A oncogene during cell transformation is sufficient to induce susceptibility to lysis by host inflammatory cells. *Proc. Natl. Acad. Sci. USA* **83,** 6965–6969.

Cook, J. L., Wilson, B. A., Wolf, L. A., and Walker, T. A. (1993). E1A oncogene expression level in sarcoma cells: An independent determinant of cytolytic susceptibility and tumor rejection. *Oncogene* **8,** 625–635.

Fox, J., Brandt, C., Wasserman, F., Hall, C., Spigland, I., Kogan, A., and Elverback, L. (1969). The virus watch program: A continuing surveillance of viral infections in metropolitan New York families. *Am. J. Epidemiol.* **89,** 25–52.

Frisch, S. M. (1991). Antioncogenic effect of adenovirus E1A in human tumor cells. *Proc. Natl. Acad. Sci. USA* **88,** 9077–9081.

Gooding, L. R., Aquino, L., Duerksen-Hughes, P. J., Day, D., Horton, T. M., Yei, S., and Wold, W. S. M. (1991). The E1B 19,000-molecular-weight protein of group C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not of mouse cells. *J. Virol.* **65,** 3083–3094.

Gooding, L. R., Elmore, L. W., Tollefson, A. E., Brady, H. A., and Wold, W. S. (1988). 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell* **53,** 341–346.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36,** 59–72.

Green, M., Wold, W., Mackey, J., and Rigden, P. (1979). Analysis of human tonsil and cancer DNAs and RNAs for DNA sequences of group C (serotypes 1,2,5 and 6) human adenovirus. *Proc. Natl. Acad. Sci. USA* **76,** 6606–6610.

Harlow, E., Franza, R., Jr., and Schley, C. (1985). Monoclonal antibodies specific for adenovirus early region 1A proteins: Extensive heterogeneity in early region 1A products. *J. Virol.* **55,** 533–546.

Jones, N., and Shenk, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17,** 683–689.

Kärre, K., Ljunggren, H. G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2 deficient lymphomas variants suggests alternative immune defence strategy. *Nature* **319,** 675–678.

Kitajewski, J., Schneider, R. J., Safer, B., Munemitsu, S. M., Samuel, C. E., Thimmappaya, B., and Shenk, T. (1986). Adenovirus VAI RNA antagonizes the antiviral action of interferon by preventing activation of the interferon-induced eIF-2 alpha kinase. *Cell* **45,** 195–200.

Koneru, B., Jaffe, R., Esquivel, C. O., Kunz, R., Todo, S., Iwatsuki, S., and Starzl, T. E. (1987). Adenoviral infections in pediatric liver transplant recipients. *J. Am. Med. Assoc.* **258,** 489–492.

Kornbluth, J., Flomenberg, N., and Dupont, B. (1982). Cell surface phenotype of a cloned line of human natural killer cells. *J. Immunol.* **129,** 2831–2837.

Lavery, D., Fu, S. M., Lufkin, T., and Chen-Kiang, S. (1987). Productive infection of cultured human lymphoid cells by adenovirus. *J. Virol.* **61,** 1466–1472.

Lederman, M., and Winkelstein, J. A. (1985). X-linked agammaglobulinemia: An analysis of 96 patients. *Medicine* **64,** 145–156.

Leff, T., and Chambon, P. (1986). Sequence-specific activation of transcription by adenovirus E1a products is observed in HeLa cells but not in 293 cells. *Mol. Cell. Biol.* **6,** 201–208.

Logan, J., Pilder, S., and Shenk, T. (1984). Functional analysis of adenovirus type 5 early region 1B. *Cancer Cells* **59,** 527–532.

Martin, K. J., Lillie, J. W., and Green, M. R. (1990). Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. *Nature* **346,** 147–152.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* **89,** 7742–7746.

Rice, S., and Klessig, D. F. (1985). Isolation and analysis of adenovirus type 5 mutants containing deletions in the gene encoding the DNA binding protein. *J. Virol.* **56,** 767–778.

Routes, J. M., and Cook, J. L. (1989). Adenovirus persistence in man: Defective E1A gene product targeting of infected cells for killing by natural killer cells. *J. Immunol.* **142,** 4022–4026.

Routes, J. M., and Cook, J. L. (1990). Resistance of human cells to the adenovirus E3 effect on class I MHC antigen expression: Implications for antiviral immunity. *J. Immunol.* **144,** 2763–2770.

Routes, J. M., Metz, B. A., and Cook, J. L. (1993). Endogenous expression of E1A in human cells enhances the effect of adenovirus E3 on class I major histocompatibility complex antigen expression. *J. Virol.* **67,** 3176–3181.

Spindler, K. R., and Berk, A. J. (1984). Rapid intracellular turnover of adenovirus 5 early region 1A proteins. *J. Virol.* **52,** 706–710.

Straus, S. E. (1984). Adenovirus infections in humans. *In* "The Adenoviruses" (H. S. Ginsberg, Ed.). Plenum, New York.

Svensson, C., and Akusjärvi, G. (1984). Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes. *EMBO J.* **3,** 789–794.

Walker, T. A., Wilson, B. A., Lewis, A. M., Jr., and Cook, J. L. (1991). E1A oncogene induction of cytolytic susceptibility eliminates sarcoma cell tumorigenicity. *Proc. Natl. Acad. Sci. USA* **88,** 6491–6495.

Wasserman, R., August, C. S., and Plotkin, S. A. (1988). Viral infections in pediatric bone marrow transplant patients. *Pediatr. Infect. Dis. J.* **7,** 109–115.

Weinberg, D. H., and Ketner, G. (1983). A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. *Proc. Natl. Acad. Sci. USA* **80,** 5383–5386.

Welsh, R. M. (1986). Regulation of virus infections by natural killer cells. *Nat. Immun. Cell Growth Regul.* **5,** 169–199.

White, E., Spector, D., and Welch, W. (1988). Differential distribution of the adenovirus E1A proteins and colocalization of E1A with the 70-kilodalton cellular heat shock protein in infected cells. *J. Virol.* **62,** 4153–4166.

White, E., and Stillman, B. (1987). Expression of adenovirus E1B mutant phenotypes is dependent on host cell and on synthesis of E1A proteins. *J. Virol.* **61,** 426–435.

Zahradnik, J., Spencer, M., and Porter, D. (1980). Adenovirus infection in the immunocompromised patient. *Am. J. Med.* **68,** 725–732.